Developmental Biology 405 (2015) 189–201

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

Talin is required to position and expand the luminal domain of the *Drosophila* heart tube

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ARTICLE INFO

Article history:
Received 19 February 2015
Received in revised form
25 April 2015
Accepted 28 April 2015
Available online 7 May 2015

Keywords:
Drosophila
Talin
Tubulogenesis
Lumen
Heart development
Actin binding domain

ABSTRACT

Fluid- and gas-transporting tubular organs are critical to metazoan development and homeostasis. Tubulogenesis involves cell polarization and morphogenesis to specify the luminal, adhesive, and basal cell domains and to establish an open lumen. We explore a requirement for Talin, a cytoplasmic integrin adapter, during *Drosophila melanogaster* embryonic heart tube development. Talin marks the presumptive luminal domain and is required to orient and develop an open luminal space within the heart. Genetic analysis demonstrates that loss of zygotic or maternal-and-zygotic Talin disrupts heart cell migratory dynamics, morphogenesis, and polarity. Talin is essential for subsequent polarization of luminal determinants Slit, Robo, and Dystroglycan as well as stabilization of extracellular and intracellular integrin adhesion factors. In the absence of Talin function, mini-lumens enriched in luminal factors form in ectopic locations. Rescue experiments performed with mutant Talin transgenes suggest that actin-binding is required for normal lumen formation, but not for initial heart cell polarization. We propose that Talin provides instructive cues to position the luminal domain and coordinate the actin cytoskeleton during *Drosophila* heart lumen development.

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Introduction

Organisms rely on tubular organs to transport fluids, gases, and cells between and through tissues. Although diverse in their size, structure, development, and function, all tubular organs have a stereotypical polarized architecture, with a basal surface interacting with surrounding tissues and a luminal, often apical, domain facing the inner space (Lubarsky and Krasnow, 2003; Datta et al., 2011). Tubulogenesis must determine the luminal site through spatial environmental and adhesion cues. These establish luminal–basal polarity, specify membrane domains, and develop the intra-luminal environment (Ferrari et al., 2008; Datta et al., 2011). While these events are interdependent, their characterization reveals generalizable principles of tube formation. Insights into the *in vitro* MDCK cyst model suggest that an early step in lumen formation is the accumulation of luminal markers such as Crumbs, Par3/aPKC, actin, and Podocalyxin at the apical membrane initial site (Schlüter et al., 2009; Bryant et al., 2010; O'Brien et al., 2002). Correct establishment of this site is dependent on spatial cues provided by the surrounding extracellular matrix through the integrin transmembrane receptor (Yu et al., 2005; O'Brien et al., 2002). Although reduced integrin function does not prevent accumulation of luminal markers, cyst polarity is inverted and Podocalyxin remains on the outer surface of the MDCK cell aggregates (Yu et al., 2005). While these mechanisms have been elegantly elucidated in the MDCK *in vitro* model, validation *in vivo* has been limited.

Here we explore the role of integrin signaling *in vivo*, during tubulogenesis of the developing *Drosophila melanogaster* embryonic heart. The *Drosophila* heart is a linear tube enveloped by parallel rows of cardial cells linked pairwise across the midline. The tubular nature of the fly heart is apparent when viewed in cross-section; the cardioblasts (CBs) in each contralateral row are crescent shaped, together wrapping into a single cell-layered tube wall enclosing a central lumen through which hemolymph is pumped. Unlike the targeted secretion hollowing mechanism which drives MDCK cyst development (Bryant et al., 2010), tubulogenesis of the *Drosophila* heart proceeds through localized establishment of cell–cell adhesions adjacent to the non-adhesive luminal membrane. Despite its relative simplicity compared to the vertebrate heart, the *Drosophila* cardiac tube provides a tractable model to study heart development, function, and aging (Bier and Bodmer, 2004; Tao and Schulz, 2007; Reim and Frasch, 2010; Seyres et al., 2012).

Key to development of the heart tube is polarization of several cell-surface factors which promote luminal domain formation and restrict the cell–cell adhesions. Interestingly, these luminal factors do not include some of the well-characterized apical epithelial markers such as Crumbs, Bazooka/Par3, nor aPKC (Qian et al., 2005; Medioni

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http://dx.doi.org/10.1016/j.ydbio.2015.04.024
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et al., 2008; Brandt and Paululat, 2013), but do include basal proteins including βPS1 integrin, Dystroglycan (Dg), and Laminin (Vanderploeg et al., 2012; Medioni et al., 2008; Hollfelder et al., 2014) and chemo-attractant or repellent ligand–receptor pairs Slit/Robo and Netrin/Frazzled/Unc5 (Santiago-Martinez et al., 2008; Albrecht et al., 2011; Macabenta et al., 2013). While this atypical polarity has been recognized (Medioni et al., 2008; Lehmacher et al., 2012), it remains unclear how these determinants act molecularly during lumen formation. The MDCK cyst model reveals that spatial cues from the ECM must be integrated with cytoplasmic polarity pathways and actin cytoskeletal dynamics in order to promote tubulogenesis; however conservation of similar mechanisms in the atypically polarized *Drosophila* heart remains to be established. Identification of a non-muscle myosin, Zipper, genetically acting upstream of Slit in the *Drosophila* heart (Vogler et al., 2014) suggests a model wherein actin dynamics mediate adhesion signals from integrin during lumen establishment.

Here we dissect the role of Talin, encoded by *rhea*, in *Drosophila* heart tubulogenesis. Talin is a cytoplasmic adapter protein which serves as a bridge between the ECM and the cytoskeleton by physically binding to integrins, actin, and several integrin adhesion-complex proteins. Through these interactions, Talin regulates integrin–ECM affinity, cytoplasmic adhesion complex assembly, and actin cytoskeletal dynamics (Das et al., 2014). We identify Talin as an early and essential marker of the lumen site and provide genetic and immunohistochemical evidence that Talin is required for orientation and polarization of luminal determinants. Furthermore, data from domain-specific mutations in Talin transgenes suggest that Talin–actin interaction is key to heart lumen formation.

Materials and methods

Drosophila strains and genetics

All strains were maintained in a *yw* background over GMR > YFP or Kr > GFP marked balancer chromosomes (BL 23230, 23231, or 5193). Multiple amorphic alleles were tested for *rhea* zygotic experiments: $rhea^{79}$ (Brown et al., 2002), $rhea^{1}$ and $rhea^{2}$ (Prout et al., 1997). $rhea^{79}$ or mys^{XG43} (Ellis et al., 2011) germ-line clone mutants lacking both maternal and zygotic Talin or βPS1 were generated using the dominant female sterile technique (Chou and Perrimon, 1996). For live imaging of the CB leading edge, dmefGal4 (Ranganayakulu et al., 1996), UAS–moesin–mcherry (Millard and Martin, 2008), tupGFP (Tokusumi et al., 2007), and $rhea^{79}$ were recombined onto a single chromosome.

The ubiquitously expressed Talin rescue transgenes were described previously: Wildtype, $IBS1^{R367A}$, $IBS2^{KS>DD}$, $IBS1^{R367A}IBS2^{LI>AA}$ (Ellis et al., 2011), $ABD^{KVK>DDD}$, and ABD^{R2531G} (Franco-Cea et al., 2010). Each transgene was outcrossed to *yw* for three generations to remove potential modifying mutations. In $rhea^{79}$ homozygous embryos expressing $Talin^{WT}$, $Talin^{IBS1-}$, $Talin^{IBS2-}$, $Talin^{ABDa-}$, or $Talin^{ABDb-}$, Talin was detected at levels above those in $rhea^{79}$ heterozygotes and Talin localization within the heart was similar to that observed in wildtype (see Fig. 2 in Supplementary material). However, trace levels of Talin protein were detected in $rhea^{79}$ embryos carrying $Talin^{IBS1-,IBS2-}$ (see Fig. 2B and G in Supplementary material), so further data is not presented.

Immunohistochemistry

Embryo fixation and staining were adapted from standard protocols (Patel, 1994). Primary antibodies used were as follows: mouse anti-βPS1 (1:30, Developmental Studies Hybridoma Bank (DSHB) DF.6G11-s), rabbit anti-CAP (1:1,000, Bharadwaj et al., 2013), mouse anti-Dlg (1:30, DHSB 4F3), rabbit anti-MEF (1:5,000, Vanderploeg et al., 2012), rabbit anti-Dg (1:100, this study), chicken anti-GFP (1:2,000, Cedarlane ab13970), mouse anti-LanA (1:20, this study), mouse anti-Prc (1:30, DSHB EC11 anti-Pericardin), rabbit anti-Pinch (1:500, Clark et al., 2003), mouse anti-Robo (1:30, DHSB 13C9 anti-Robo-s), mouse anti-Slit (1:30, DHSB C555.6D-s), mouse anti-Talin (1:30, DHSB Talin E16B-s), and rat anti-Zfh1 (1:150, Lai et al., 1991). Dg antiserum was raised in New Zealand white rabbits against C-terminal GST-Dg (Deng et al., 2003). The monoclonal LanA antibody was generated against the ANTEHDHIDYSV peptide, corresponding to amino acids 59–70 of LanA (Abmart, China). Alexa 488, 546, 594, and 647 secondary antibodies were used at a 1:150 dilution (Molecular Probes). Images were acquired using a Leica SP5 confocal microscope. Transverse images and frontal images displaying anti-Dg are single optical sections, while all other frontal images are projections of four to six optical sections. Images were processed using ImageJ and assembled with Adobe Photoshop.

Live embryo imaging

Live imaging of embryos was performed using the hanging drop method (Reed et al., 2009). Images were acquired every two minutes using a Leica SP5 confocal microscope. Stacks of 20–30 images 1 μm apart were projected. For analysis of filopodia density, the number of extended filopodia was counted for each six-cell hemi-segment within 13 μm of the midline. Statistical differences were compared using the Student's *t*-test.

Western blot analysis

For the Western blots shown in Fig. 2 of Supplementary material, ten stage 17 embryos per genotype were individually selected using –GFP or –YFP marked chromosomes. Membranes were probed with primary antibodies mouse anti-Talin (1:500, DHSB Talin E16B) or anti-βTubulin (1:1,000, DHSB E7) and horseradish peroxidase-conjugated anti-mouse secondary antibody (1:5,000, Jackson ImmunoResearch 115-035-166). Western blots were analyzed and quantified using ImageJ, with βTubulin used as a loading control reference. Graph images show the average band intensity values (relative to $rhea^{79}$ heterozygote levels) of 5–7 independent Western blots.

Results

Talin is an early marker of the lumen initiation site

Following *Drosophila* cardial cell specification, the cells align in two lateral rows adjacent to the amnioserosa, an extraembryonic epithelial tissue which transiently covers the dorsal surface of the embryo. The cardial cells include the CBs, which eventually form the lumen-enclosing heart wall, and the flanking pericardial cells (Fig. 1A–C, green and blue respectively). During dorsal closure, the overlying ectoderm migrates towards the dorsal midline to replace the amnioserosa-filled dorsal layer. Coincident with this process, through embryonic stages 14–17, the CBs and pericardial cells collectively move towards the dorsal midline and complete tubulogenesis (Fig. 1A–C and F–H). As integrin function is required for polarization of CBs, we asked whether Talin, a critical component of the integrin adhesion complex, apicalizes during heart development.

Throughout CB migration, Talin immunolabel was detected along the leading and basal CB domains (Fig. 1A and B arrow and arrowheads respectively), but was not detected on lateral cell membrane between ipsilateral CBs. Talin immunolabel was not restricted to the heart, but was also detected in surrounding tissues including the amnioserosa and gut (Fig. 1A and B). Following

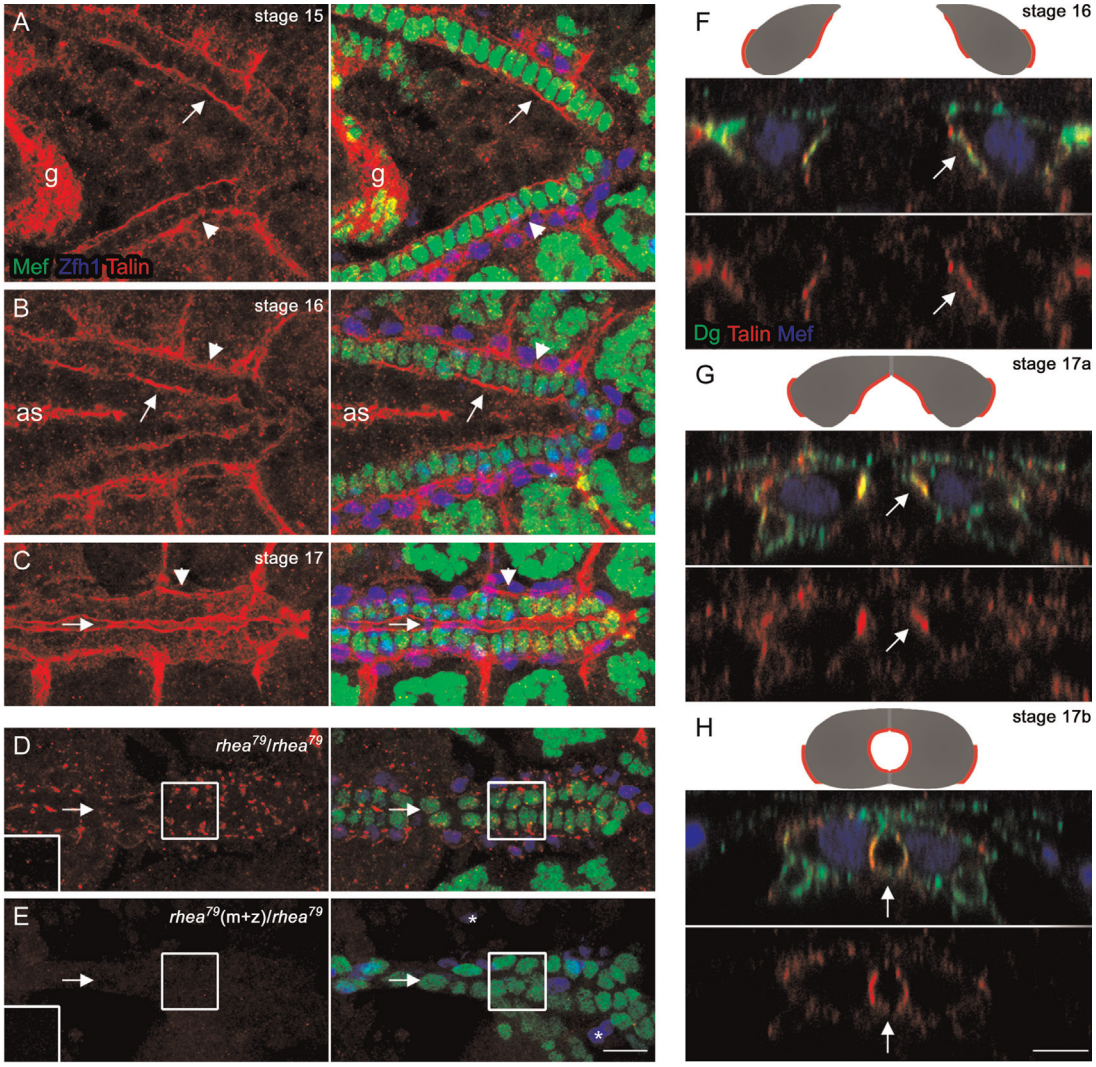


Fig. 1. Talin localization in the developing *Drosophila* embryonic heart. (A–E) Frontal images of the hearts of wildtype and *rhea* null *Drosophila* at stage 15 (A), 16 (B), and 17 (C–E) of embryogenesis. During cardioblast migration (A, B) and through lumen enclosure (C), Talin (red) is localized along the advancing apical domain (arrow) and the trailing basal surface along the CB-pericardial cell interface (arrowhead), but is excluded laterally. Talin immunolabel can also be detected in the gut (g) and amnioserosa (as). Relative to similar-staged wildtype (C), *rhea* zygotic mutants have reduced Talin, with low levels detected in punctate accumulations along the apical and basal cardioblasts domains (D). In mutant embryos lacking both maternal and zygotic Talin (E), no Talin immunolabel is detected and Zfh-1 expressing pericardial cells are frequently detached from the CB basal surface and displaced laterally (asterisks). Panels representing mutant embryos were imaged with adjusted gain to visualize low intensity immunolabel signals. Insets were imaged at the same settings as wildtype. (F–H) Transverse images of the *Drosophila* heart at stages 16 and 17 of embryogenesis. During cardial cell migration, the triangular cardioblasts (outlined with Dg) extend dorsally towards the midline. Talin immunolabel is concentrated ventral to the leading edge (arrow), marking the pre-luminal domain (F and G). Following migration, the cardioblasts adopt a crescent shape to enclose the Talin-lined lumen (H). CB nuclei here and in subsequent figures are labeled with αMEF (green in A–E, blue in F–H). Pericardial cells are labeled with αZfh1 (blue). In all figures posterior of the heart is to the right in frontal images, dorsal is to the top in transverse sections. Calibration: 10 μm (A–E), 5 μm (F–H).

migration, Talin accumulated at the basal surface and apically, at the midline (Fig. 1C).

Recruitment or stabilization of Talin at the luminal and basal surface is βPS1 (*mys*) dependent (see Fig. 1 in Supplementary material), consistent with Talin binding to the β-integrin cytoplasmic tail at sites of integrin adhesion (Calderwood et al., 2002; Rodius et al., 2008). Embryos zygotically homozygous for the null mys^{XG43} allele had reduced Talin immunolabel along the midline and instances of diffuse accumulation on lateral CB membranes (see Fig. 1B and C in Supplementary material). In embryos that lacked both zygotic and maternally deposited βPS1 (see methods), there was no consistent apical or basal pattern of Talin accumulation (see Fig. 1D in Supplementary material).

Although previously termed the "apical" domain (Fremion et al., 1999; Chartier et al., 2002; Santiago-Martinez et al., 2008), the CB surface facing the midline (arrow in Fig. 1C) can be functionally divided into a central luminal domain sealed dorsally and ventrally by two junctional or adherent regions (Medioni et al., 2008). In the stage 17 embryonic heart, a transverse optical section revealed Talin accumulation along the luminal domain (arrow) and excluded from the junctional domain (Fig. 1H). However, Talin was localized to the luminal domain well before tubulogenesis occurs. During CB migration, Talin accumulated along the pre-luminal or lumen initiation site (Fig. 1F and G). This is a pattern reminiscent of βPS1 integrin localization (Vanderploeg et al., 2012), suggesting that Talin, like βPS1 integrin, is an early site-determinant for lumen formation.

Maternal Talin is sufficient for cardioblast–pericardial cell attachment

Previous studies of *Drosophila* Talin reported that maternally contributed Talin persists through embryogenesis (Brown et al., 2002). We asked if maternal Talin is present in the developing

heart and, furthermore, whether it is sufficient for heart tubulogenesis. Compared to wildtype, zygotic *rhea*^{79} mutant embryos had markedly reduced Talin immunolabel (Fig. 1D inset); however, by increasing the detection sensitivity, low levels of Talin immunolabel were still apparent in basal and apical punctate accumulations (Fig. 1D). Similar results were obtained when additional null alleles (*rhea*^{1} or *rhea*^{2}) and transheterozygote combinations were tested (see Fig. 1E–H in Supplementary material). In contrast, mutant germ-line clone *rhea*^{79} embryos lacked both maternal and zygotic Talin (m+z) and had no detectable Talin (Fig. 1E).

Although in zygotic mutants Talin is markedly reduced compared to wildtype, the residual maternal Talin has a significant impact on embryogenesis. Maternal and zygotic *rhea* mutant embryos, but not zygotic mutants, have developmental defects in the embryonic ectoderm and amnioserosa (Prout et al., 1997; Brown et al., 2002; Ellis et al., 2013). In the heart, unlike wildtype

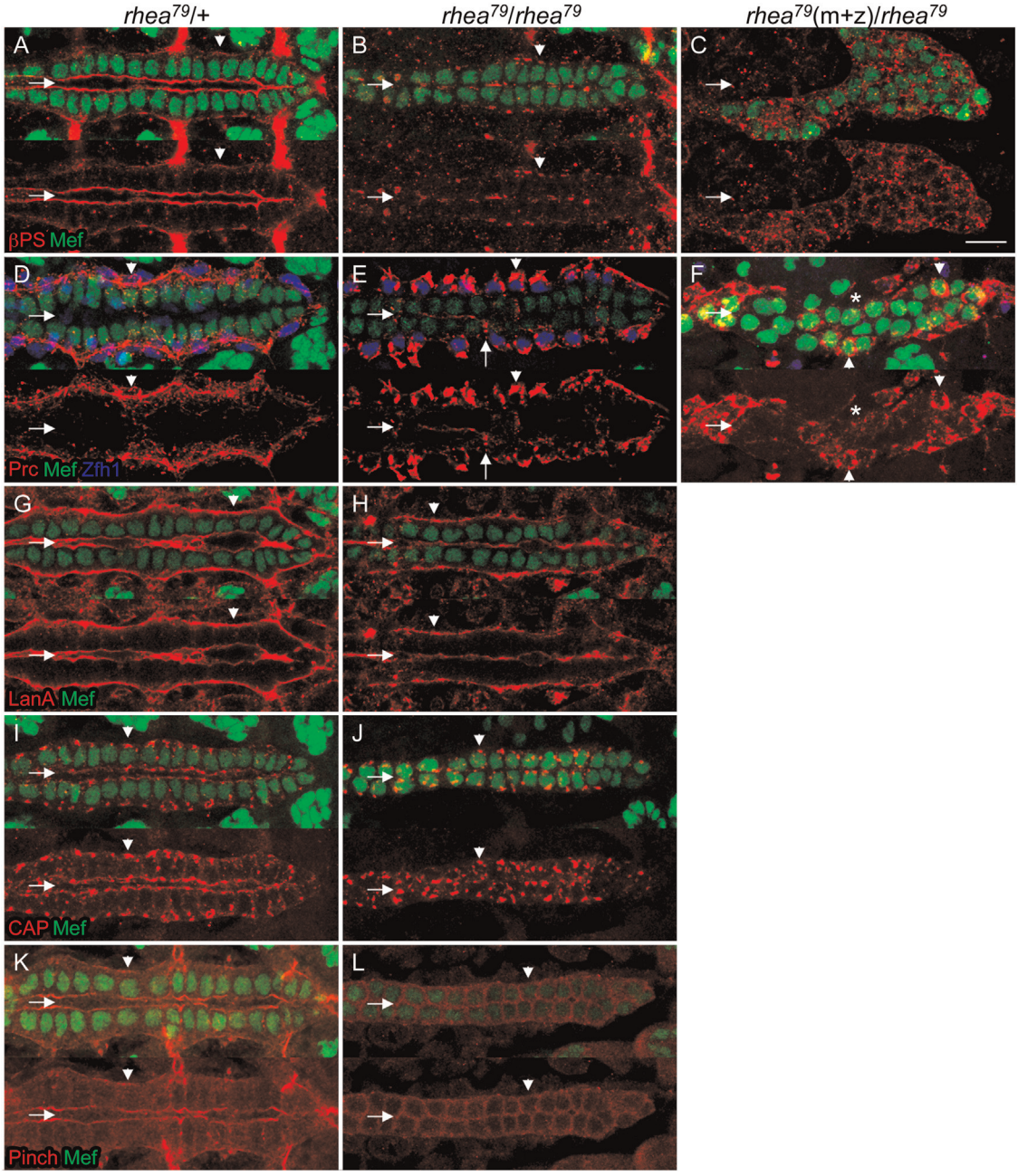


Fig. 2. ECM components and cytoplasmic integrin adhesome proteins are abnormally distributed in *rhea* mutant embryos. In *rhea* heterozygous embryos (A), βPS1 immunolabel is detected at high levels along the midline luminal surface (arrow) and at lower levels basally (arrowhead). *rhea* mutant embryos have reduced βPS1 that accumulates in a punctate (zygotic nulls, B) or diffuse (maternal and zygotic nulls, C) localization pattern, respectively. Prc is restricted to CB basal surface in *rhea* heterozygotes (D), along the CB-pericardial cell interface (arrowhead). In zygotic *rhea* mutant embryos (E), Prc remains basal, often surrounding the pericardial cells (arrowhead), but is also detected apically (horizontal arrow) and laterally (vertical arrow) between neighboring CBs. Maternal and zygotic *rhea* mutant embryos have severely disrupted Prc distribution (F). Basal accumulation is frequently interrupted and non-continuous (arrowhead), while mislocalized Prc completely surrounds individual CBs (arrow). In heterozygous *rhea* embryos, LanA accumulates contiguous to the apical and basal CB surfaces (G). *rhea* homozygotes have reduced LanA, most notably along the apical CB surfaces (H). Both *rhea* heterozygous and homozygous embryos have punctate accumulations of CAP present apically and basally, although fewer apical accumulations are visible in mutant embryos (I and J).The pattern of Pinch in *rhea* heterozygotes is highly polarized, with strong apical and basal immunolabel and low levels laterally (K). In contrast, Pinch is diffusely distributed around each CB in *rhea* null embryos (L). Both heterozygotes and mutants were imaged using identical settings. Arrows indicate the midline, arrowheads the CB basal surface. Calibration: 10 μm.

or zygotic *rhea* mutants (Fig. 1C and D), embryos lacking both maternal and zygotic Talin exhibited displacement of pericardial cells from the CB basal surface (asterisks, Fig. 1E). This suggests that Talin is essential for CB-pericardial cell attachment and that low maternal levels are sufficient to maintain adhesion (Fig. 1D). Taken together, these embryonic phenotypes also highlight the importance of assessing maternally and zygotically *rhea* null embryos to gain a complete understanding of Talin's function in *Drosophila* heart development. However, analysis of the heart in these embryos is complicated by the developmental defects in the surrounding ectoderm and amnioserosa which indirectly disrupt the heart (data not shown). Therefore, to thoroughly examine the role of Talin in *Drosophila* heart development, we assessed tubulogenesis in embryos retaining low, maternally provided, Talin (zygotic mutants) and in embryos completely devoid of Talin (maternal and zygotic mutants).

βPS1 integrin is disrupted in rhea mutant embryos

Studies in both vertebrates and flies suggest that Talin is required for focal adhesion assembly and stabilization of integrins at sites of adhesion (Liu et al., 2011; Brown et al., 2002). Wildtype hearts had βPS1 integrin at the luminal surface and lower levels basally (Fig. 2A). In contrast, in *rhea* mutants βPS1 levels were reduced and localization was disrupted (Fig. 2B and C). In zygotic *rhea* mutants, βPS1 integrin accumulated in a punctate pattern, similar to that seen with maternal Talin localization (Fig. 2B, compare to Fig. 1D). Maternal and zygotic *rhea* mutant embryos had a more diffuse pattern of βPS1 (Fig. 2C), consistent with Talin being required for integrin stabilization during lumen formation.

Talin is required for normal localization of extracellular and intracellular integrin-related proteins

One role of integrins is to promote proper establishment and maintenance of the ECM. For example, Laminin polymerization and ECM anchorage, crucial initiating steps in basement membrane assembly, are facilitated by integrin binding (Colognato et al., 1999; Yurchenco, 2011). We sought to determine whether Talin is similarly required to localize ECM factors surrounding the heart. Laminin A (LanA), a likely αPS3βPS1 ligand (Stark et al., 1997), is a heterotrimeric ECM glycoprotein. In wildtype embryos LanA localization mirrored that of βPS1 and Talin with strong immunolabel detected along the apical and basal CB surfaces (Fig. 2G). In *rhea* zygotic mutant embryos, LanA was reduced or absent in the apical domains (arrow, Fig. 2H) and detected at lower levels along the basal domain (arrowhead, Fig. 2H). A second *Drosophila* heart ECM component is the Type IV collagen-like Pericardin (Prc), secreted by the pericardial cells and seven-up CBs (Singhal and Martin, 2011; Chartier et al., 2002). Unlike LanA, Prc was excluded from the apical CB surface in wildtype embryos (arrow, Fig. 2D). Rather, Prc was restricted to the basal surface, lining the CB-pericardial cell interface (arrowhead, Fig. 2D), consistent with the demonstrated requirement of Prc for pericardial cell attachment (Chartier et al., 2002; Drechsler et al., 2013). This pattern was disrupted in *rhea* mutant embryos (Fig. 2E and F). In zygotic mutants, Prc was mislocalized, often found surrounding the pericardial cells (arrowhead) and present apically (horizontal arrow) and laterally (vertical arrow) within the heart (Fig. 2E). In maternal and zygotic mutant embryos, this phenotype was more severe, as Prc surrounded numerous CBs (arrowheads) and was absent from much of the basal CB surface (asterisks, Fig. 2F). This is consistent with Talin being required for CB-pericardial adhesion, a process for which maternal Talin is sufficient (Fig. 1D). In contrast, maternal Talin is not sufficient to properly establish the ECM along the lumen-forming apical surface, as LanA was reduced and Prc was ectopically deposited in *rhea* zygotic mutant embryos.

To determine if assembly of the integrin adhesome in the heart is dependent on Talin, we assessed the localization pattern of Pinch and Cbl-associated protein (CAP), two cytoplasmic proteins recruited by Talin to integrin-mediated muscle attachment sites in *Drosophila* (Clark et al., 2003; Zervas et al., 2011; Bharadwaj et al., 2013). In *Drosophila* musculature, CAP is gradually recruited to muscle attachment sites where it is required for adhesion stabilization (Bharadwaj et al., 2013). Similarly, during early stages of heart cell migration, CAP immunolabel was initially weak, but increased as heart development proceeded (data not shown). By late stage embryogenesis, CAP lined the basal and apical CB surface in a punctate pattern (Fig. 2I). CAP overlapped with Talin immunolabel, but was more restricted (compare Fig. 2I with Fig. 1C), suggesting that Talin is not sufficient to recruit CAP. In addition, CAP localization was not sensitive to reduced Talin levels: in *rhea* zygotic mutants, the punctate apical and basal pattern of CAP remained, despite perturbations in the heart itself (Fig. 2J). In contrast, localization of Pinch, an integrin adhesion scaffold protein, was Talin dependent. Pinch was recruited to sites of βPS integrin and Talin, accumulating most prominently along the apical and basal CB surfaces (Fig. 2K; Clark et al., 2003). In *rhea* zygotic mutants, Pinch was no longer restricted in its localization, but it was found more diffusely within each CB (Fig. 2L). Taken together, it appears that Talin is required for assembly of the early integrin adhesome (Pinch), but is dispensable and insufficient for recruitment of stable adhesion components (CAP).

Loss of Talin disrupts cardioblast polarity

In the mature embryonic heart the luminal determinants Slit and Robo accumulate in the lumen, along the apical midline surface of the CBs (Fig. 3A, B, F and G; Qian et al., 2005; MacMullin and Jacobs, 2006; Santiago-Martinez et al., 2008). We previously established that αPS3βPS1 integrin stabilizes Slit and Robo within the heart domain to promote tube formation (Vanderploeg et al., 2012). We hypothesized that integrins mediate heart development through the integrin adhesion complex. If true, we expect that loss of Talin would recapitulate the phenotype of *scab* (αPS3) and *mys* (βPS1) null mutations. Supporting this hypothesis, both zygotic *rhea* mutants and maternal and zygotic *rhea* nulls were characterized by a loss of apical Slit and Robo (horizontal arrow, Fig 3C, E, H and J). Instead, Slit and Robo accumulated in small lateral pockets between ipsilateral CBs or apically at tetra-cellular intersections (vertical arrows, Fig. 3C, E, H and J), where they colocalized with Dg (vertical arrows, Fig. 3D and I). Dg, a membrane component of the conserved Dystrophin complex, accumulated along the luminal and basal CB surfaces in wildtype embryos (Fig. 3B and G, Medioni et al., 2008). Significantly, in both wildtype and mutant embryos, Dg localization in the heart did not overlap with the lateral junctional marker Discs-large (Dlg). In wildtype embryos, Dlg was absent from the midline lumen. Instead, it was detected primarily along the lateral adhesions between ipsilateral CBs (Fig. 3K, Medioni et al., 2008). In contrast, in *rhea* mutants Dlg was observed along the midline between the contralateral CB rows (Fig. 3L) but was excluded from the ectopic Dg-rich lateral sites (arrows, Fig. 3L and M). Thus ectopic lateral pockets are reminiscent of the wildtype Robo- and Dg-rich midline lumen both in molecular composition and in the inhibition of adhesion. Taken together, it appears that Talin is not required for co-localization of Slit, Robo, or Dg to generate non-adhesive cell surface domains, but Talin is required to restrict these known luminal determinants to the apical CB surface.

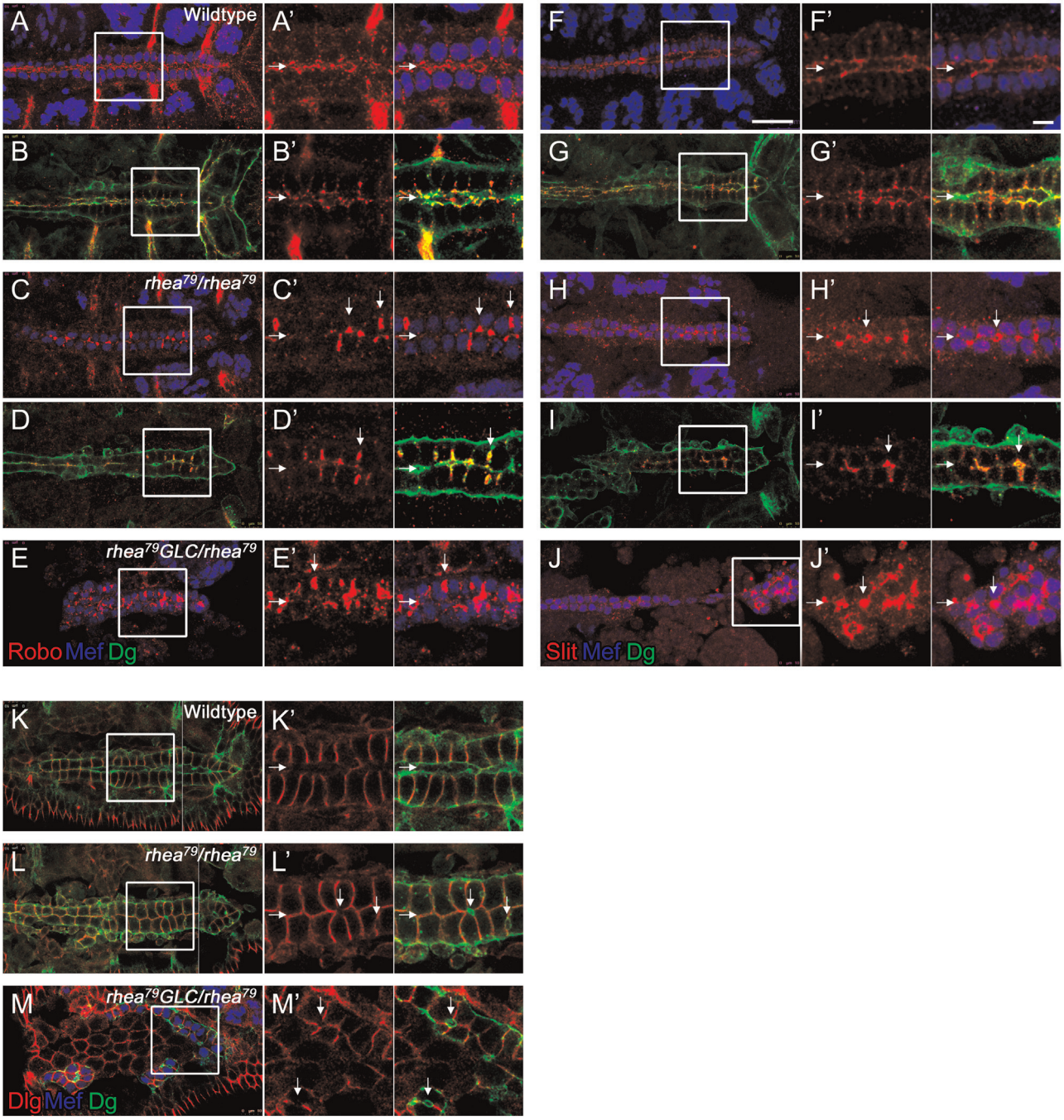


Fig. 3. *rhea* mutants have ectopic lateral lumina. In stage 17 wildtype embryos, Robo and Slit localize apically, along the midline between the cardioblast rows (arrows, A and F) where they colocalize with Dg (B and G). Zygotic *rhea* mutant embryos are characterized by a loss of apical Robo and Slit, with punctate accumulations detected laterally or at the site of tetra-cellular intersections (arrowheads, C and H). These punctate accumulations of Slit and Robo overlap with Dg-rich ectopic open pockets (D and I). In maternal and zygotic *rhea* mutants, the punctate Robo and Slit accumulations are similar to those in zygotic mutants, although the overall heart structure is more severely disrupted (E and J). These ectopic lateral pockets have characteristic luminal appearance: they accumulate luminal domain markers Slit, Robo, and Dg, and they lack the junctional Dlg. In wildtype embryos, Dlg labels the lateral cardioblast surfaces, but is absent from the Dg-rich midline luminal surface (K). In *rhea* mutant embryos, Dlg lines the midline (horizontal arrow) and is present along much of the lateral cardioblast surface, but is excluded from the Dg-rich ectopic lumina (vertical arrows, L and M). Right panels are enlarged images of framed sections on the left. Calibration: 20 μm.

Talin is required for formation of an open lumen

Prior to reaching their contralateral partners at the dorsal midline, migrating CBs begin a series of coordinated changes in cell shape required for complete enclosure of the lumen (Medioni et al., 2008). As dorsal closure commenced, the increasingly triangular-shaped CBs followed the leading ectodermal cells, protruding dorsally towards the midline (Fig. 4A and B). Upon completion of dorsal closure, the CBs migrated between the ectoderm and the internalizing amnioserosa and adhered dorsally to the opposing CBs (Fig. 4C). Following the initial dorsal attachment, the CBs adopted a crescent shape and adhered ventrally to fully enclose the lumen (Fig. 4D). Dg immunolabel demarcated the lumen boundaries (green, above arrow), while short dorsal and ventral regions of adhesion enclosing the lumen were identified by Dlg accumulation (red, Fig. 4D).

We assessed heart development in embryos devoid of Talin. In maternal and zygotic *rhea* mutant embryos, tubulogenesis failed in a

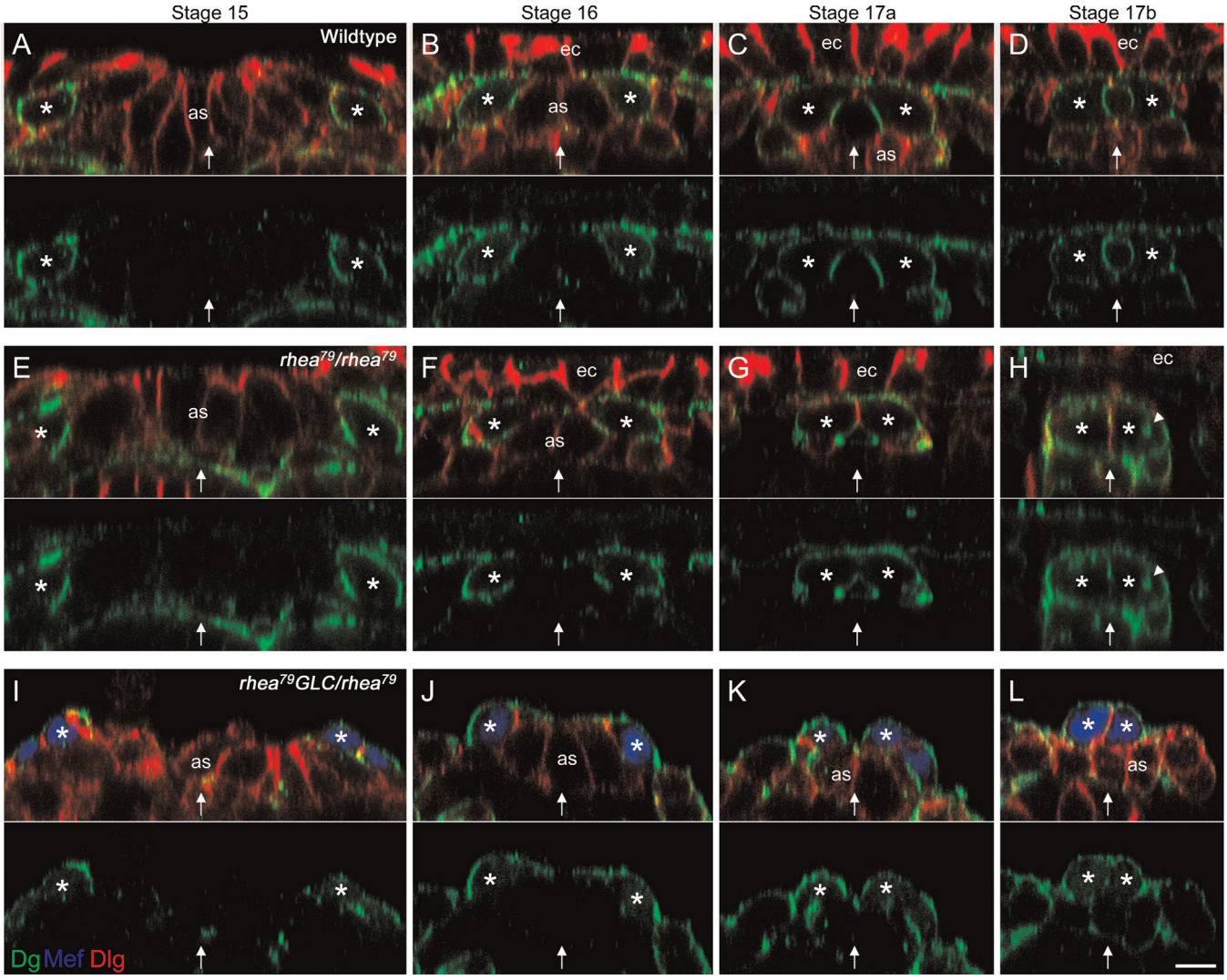


Fig. 4. Talin is essential for lumen formation. Transverse images of the dorsal midline at stage 15, stage 16, and early and late stage 17 of embryogenesis. In wildtype embryos (A–D), migrating CBs become triangular-shaped and extend dorsally towards the midline (stage 15, 16), eventually adhering (stage 17a) and enclosing a lumen (stage 17b). The midline lumen (above arrow in D, outlined by Dg) is sealed by dorsal and ventral junctions (in red, Dlg, above and below the lumen). Early in migration, CBs in *rhea* zygotic mutants frequently adopt a triangular shape similar to those in wildtype embryos (E and F). However, rather than continue to extend a narrow dorsal protrusion towards the midline, CBs are typically more rounded (F and G) and contact contralateral partners along a large dorsal region (red above vertical arrow; G). Following migration, the CBs do not adopt a crescent shape to enclose a midline lumen, but have an extended junctional domain (Dlg, red) at the midline between opposing cardioblasts (H). Arrowheads identify ectopic lateral lumens (H). In maternal and zygotic *rhea* mutant embryos (I–L), CBs are abnormally shaped throughout migration and no lumen forms at the midline (arrow). These mutant embryos additionally have ectoderm closure (ec) and amnioserosa (as) defects (I–L). Arrowheads indicate the midline. Asterisks identify the CBs. Calibration: 10 μm.

manner similar to that previously described for integrin *scab* or *mys* mutants (Vanderploeg et al., 2012). During migration, the CBs did not adopt the expected triangular profile extending towards the midline and lacked a Dg-rich pre-luminal domain (Fig. 4I–K). Once contralateral CBs met, they were unable to form a crescent morphology to enclose a lumen, but rather remained rounded and formed an extended domain of adhesion (Fig. 4L). However, since germ-band retraction and dorsal closure are severely disrupted in embryos devoid of both maternally and zygotically expressed Talin (Brown et al., 2002; Ellis et al., 2011), it remained unclear if the heart tubulogenesis failure was caused indirectly by disruptions in the surrounding ectoderm and amnioserosa tissues. To address this question, we assessed heart development in *rhea* zygotic mutants in which the residual maternal expression of Talin is sufficient to maintain the structure of the ectoderm and amnioserosa. During migration, the CBs in *rhea* zygotic mutant embryos did adopt a triangular shape similar to that observed in wildtype (Fig. 4E and F) suggesting that maternal Talin may be sufficient for initial polarized CB behavior. However, following migration, CBs frequently formed a larger, Dlg-enriched, dorsal attachment site and did not become crescent shaped (Fig. 4G). Rather than an open Dg-lined midline lumen, ectopic pockets of Dg were detected within the CBs rows near the lateral cell membrane (arrowhead) and the midline was marked by a continuous adherent region (arrow; Fig. 4H). Taken together, these data indicate that Talin is essential for CB polarity and the localization of lumen determinants.

Talin is required for filopodia activity during cardioblast migration

During the later stages of CB migration, the CBs extend highly dynamic membrane processes towards the dorsal midline (Haack et al., 2014; Vanderploeg et al., 2012). These leading edge membrane protrusions, characteristic of migrating cells (reviewed by Ridley (2011), can be visualized using fluorescent mCherry fused to a fragment of Moesin, an actin–plasma membrane cross-linker protein (Millard and Martin, 2008). CBs in wildtype and heterozygous *rhea*

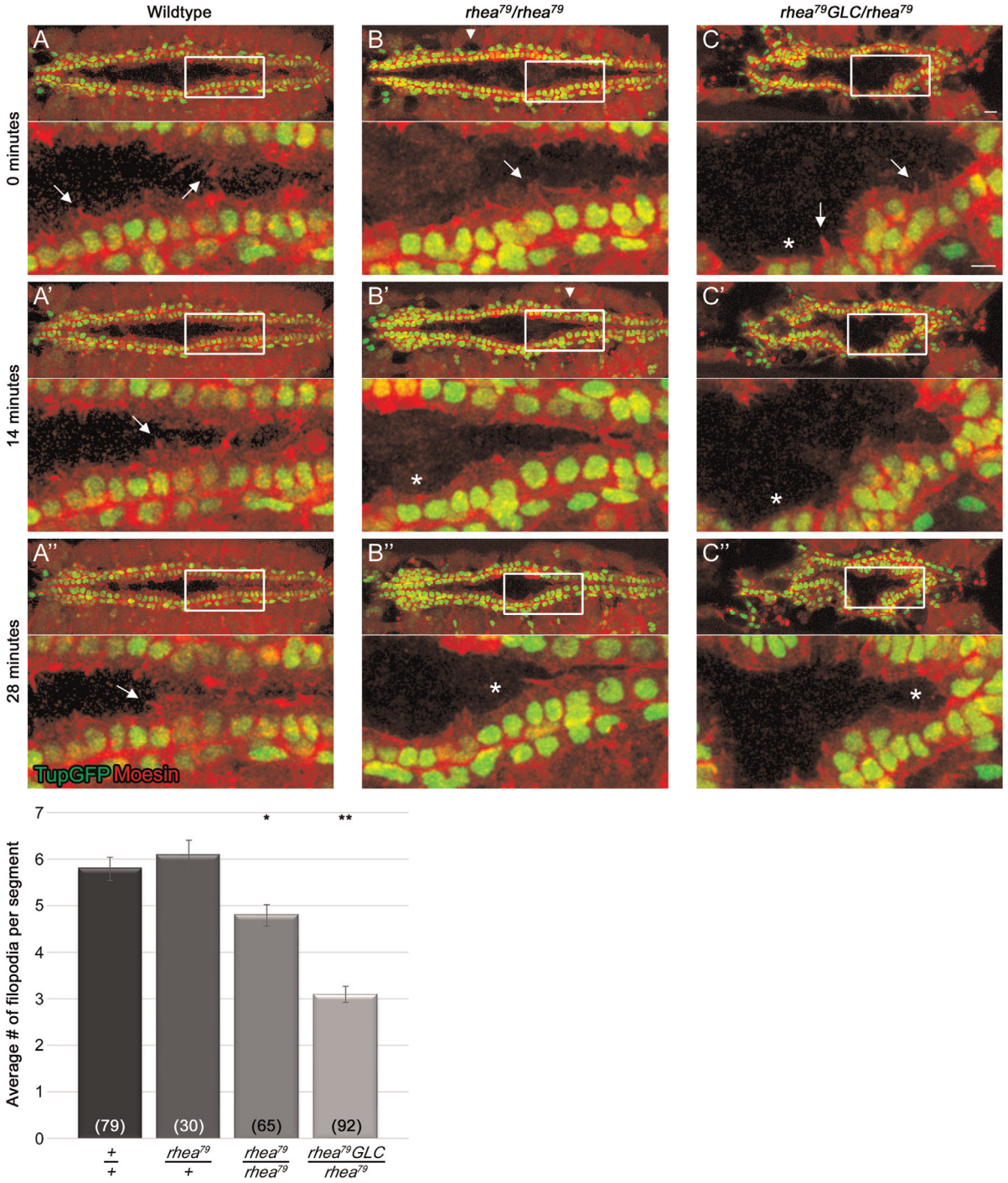
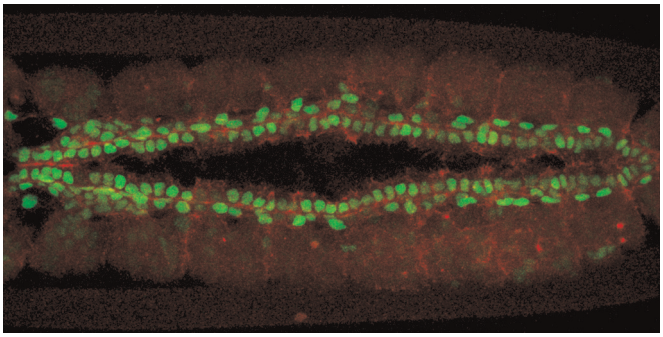
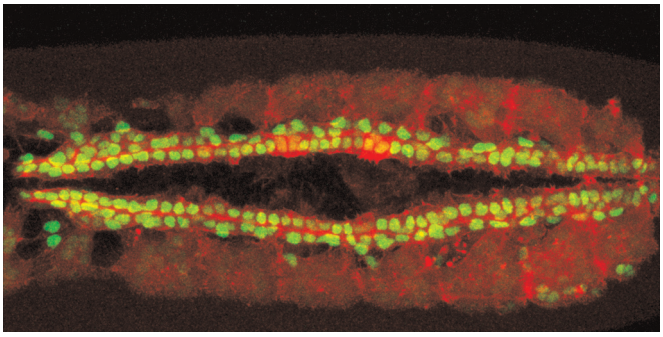


Fig. 5. Talin is required for maximal membrane leading edge activity. Live imaging of the CB leading edge visualized with dMEF-Gal4 regulated UAS–moesin–mCherry (red) and cardiac nuclei marker tup-GFP (green). In wildtype embryos (A–A″), numerous filopodia protrusions are extended towards the midline (arrows) and they dynamically extend and retract as the CBs migrate (compare 0, 14, and 28 min). In *rhea* zygotic (B–B″) or *rhea* maternal and zygotic (C–C″) mutant embryos, there are fewer protrusions extended towards the midline (arrows) and large regions of membrane quiescence (asterisks). Calibration: 15 μm. (D) Quantification of the number of filopodia extensions per six-cell segment of the heart proper. *rhea* heterozygotes have a similar number of filopodia compared to wildtype, while zygotic *rhea* and maternal and zygotic *rhea* null embryos have fewer filopodia. Since the number of filopodia increases as the distance to the midline decreases (J.V., unpublished observations), only segments within 13 μm of the midline were scored. Numbers within each column bar designate sample size in number of segments. *p*-values were calculated using an unpaired *t*-test ($^{*}p < 0.01$, $^{**}p < 1.0 \times 10^{-13}$ compared to either wildtype or *rhea*/+). Plus–minus bars represent s.d.

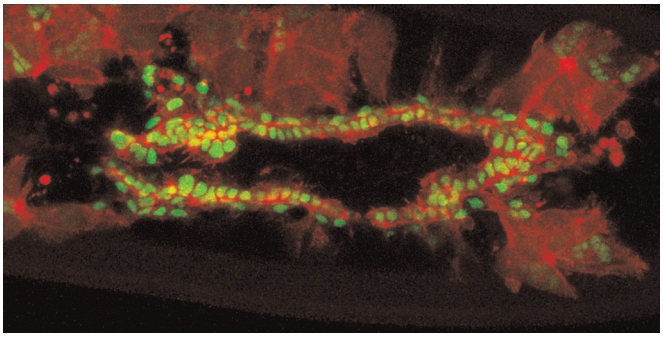
embryos rapidly extended and retracted numerous filopodia (arrows, Fig. 5A–A″, see Movie 1 in Supplementary material). In contrast, embryos lacking zygotic or maternal and zygotic Talin had reduced leading edge activity; fewer filopodia were generated and there were regions of quiescent membrane (asterisks, Fig. 5B–C″, see Movie 2 and 3 in Supplementary material). Notably, although reduced, there was residual membrane activity even in maternal and zygotic *rhea* mutants (arrows), suggesting that there may be partially redundant or compensating factors that promote leading edge activity.



Video S1. Time-lapse recording of leading edge membrane activity in migrating CBs in wildtype embryos (as summarized in Fig. 5A–A″). Note the dynamic membrane protrusions extending from the CB leading edge towards the dorsal midline. In this and subsequent movie files CB and pericardial cell nuclei are visualized with tupGFP (green) with dMEF-Gal4 regulated UAS–moesin–mCherry (red). Z-stacks were imaged every 2 min and projected maximally. Movies are displayed at 3 frames (6 min time-lapse) per second. Posterior of the heart is to the right.Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2015.04.024.



Video S2. Time-lapse recording of leading edge membrane activity in migrating CBs in *rhea* zygotic null embryos (as summarized in Fig. 5B–B″).Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2015.04.024.



Video S3. Time-lapse recording of leading edge membrane activity in migrating CBs in maternal and zygotic *rhea* null embryos (as summarized in Fig. 5C–C″). Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ydbio.2015.04.024.

Neither IBS1 nor IBS2 mediated integrin binding is essential for Talin's role in tubulogenesis

In *Drosophila* and vertebrates, Talin binds to integrin via two distinct binding sites (IBS1 and IBS2). IBS1 and IBS2 are redundant in their ability to bind Talin to integrins: Talin lacking either a functional IBS1 or IBS2 ($Talin^{IBS1-}$ or $Talin^{IBS2-}$ respectively), but not both simultaneously ($Talin^{IBS1-,IBS2-}$), were properly recruited to integrin adhesions (Ellis et al., 2011). However, in the *Drosophila* embryonic musculature, neither $Talin^{IBS1-}$ nor $Talin^{IBS2-}$ was sufficient for the maintenance of integrin adhesion during muscle contractions, although either was adequate for initial localization of ECM and integrin adhesome components (Tanentzapf and Brown, 2006; Ellis et al., 2011). During germ-band retraction and dorsal closure which require rapidly changing integrin adhesions, Talin's IBS2 domain, but not IBS1, was sufficient for normal development (Ellis et al., 2011).

To dissect the requirement for each of these integrin binding sites in heart development, we performed rescues of *rhea* homozygotes by expressing Talin transgenes with mutations designed to abrogate the integrin-binding function of each site (Ellis et al., 2011). Consistent with a redundant integrin-binding role, $Talin^{IBS1-}$ and $Talin^{IBS2-}$ were abundantly expressed and properly recruited to the apical and basal CB surfaces (see Fig. 2 in Supplementary material). Furthermore, these mutated Talin proteins were able to restore βPS1 stabilization within the heart (Fig. 6A–D). In contrast to the weak βPS1 immunolabel in *rhea* mutants (Fig. 6A), mutants expressing the $talin^{WT}$ transgene had robust βPS1 immunolabel along the apical CB surfaces (arrow, Fig. 6B). Similarly, Talin lacking either IBS1 or IBS2 was sufficient to restore βPS1 along the luminal CB domain (Fig. 6C and D). A Talin transgene mutant in both IBS1 and IBS2 domains was expressed at trace levels (see Fig. 2 in Supplementary material) precluding a direct test of complementation between the IBS domains.

In *Drosophila* heart development, the CBs undergo a dynamic morphogenesis and become increasingly polarized. Therefore, we hypothesized that similar to germ-band retraction and dorsal closure, heart tubulogenesis would require IBS2, but not the strong-adhesion mediating IBS1. Consistent with Talin being required for lumen formation, $Talin^{WT}$ was able to rescue the loss-of-lumen phenotype of *rhea* zygotic mutant embryos. Whereas *rhea* zygotic mutants had continuous Dlg-rich adhesion between opposing CBs, those expressing $Talin^{WT}$ had an open lumen lined with Dg (Fig. 6G and H, Table 1). Furthermore, as hypothesized, $Talin^{IBS1-}$ was also able to rescue lumen formation (Fig. 6I, Table 1). However, in contrast to the requirement of IBS2 during germ-band retraction and dorsal closure, $Talin^{IBS2-}$ was sufficient to promote integrin-dependent lumen formation (Fig. 6J, Table 1). These data suggest a mechanism whereby no single integrin binding domain is essential in the heart, but either of these domains is capable of mediating sufficient Talin–integrin interaction to enable heart tubulogenesis.

Direct Talin–actin binding promotes cardioblast polarization during heart tubulogenesis

Talin mediates the integrin–actin linkage either directly through several actin binding domains (ABDs; McCann and Craig, 1997; Lee et al., 2004) or indirectly by recruiting other actin-binding proteins like Vinculin and Wech (Ziegler et al., 2008; Fillingham et al., 2005; Goult et al., 2013; Löer et al., 2008). Talin's C-terminal ABD (Gingras et al., 2008; Smith and McCann, 2007) is particularly important for transient integrin adhesions during development (Franco-Cea et al., 2010; Jiang et al., 2003; Klapholz et al., 2015). We therefore hypothesized that this ABD is essential for heart tubulogenesis, as this process requires dynamic actin cytoskeleton rearrangements as the CBs enclose the lumen. To test this hypothesis, we performed rescue experiments of *rhea* zygotic mutants using two previously characterized actin-binding deficient Talin transgenes (Franco-Cea et al., 2010). When ubiquitously expressed in *rhea* zygotic mutants, both $Talin^{ABDa-}$ and $Talin^{ABDb-}$ localized to the heart midline (see Fig. 2E and F in Supplementary material) and restored the wildtype pattern of βPS1 integrin accumulation along the CB apical and basal surfaces (Fig. 6E and F). This is consistent with *Drosophila* $Talin^{ABD}$ not being required for integrin stabilization or for the recruitment of cytoplasmic integrin adhesome factors (Franco-Cea et al., 2010). Despite the correct

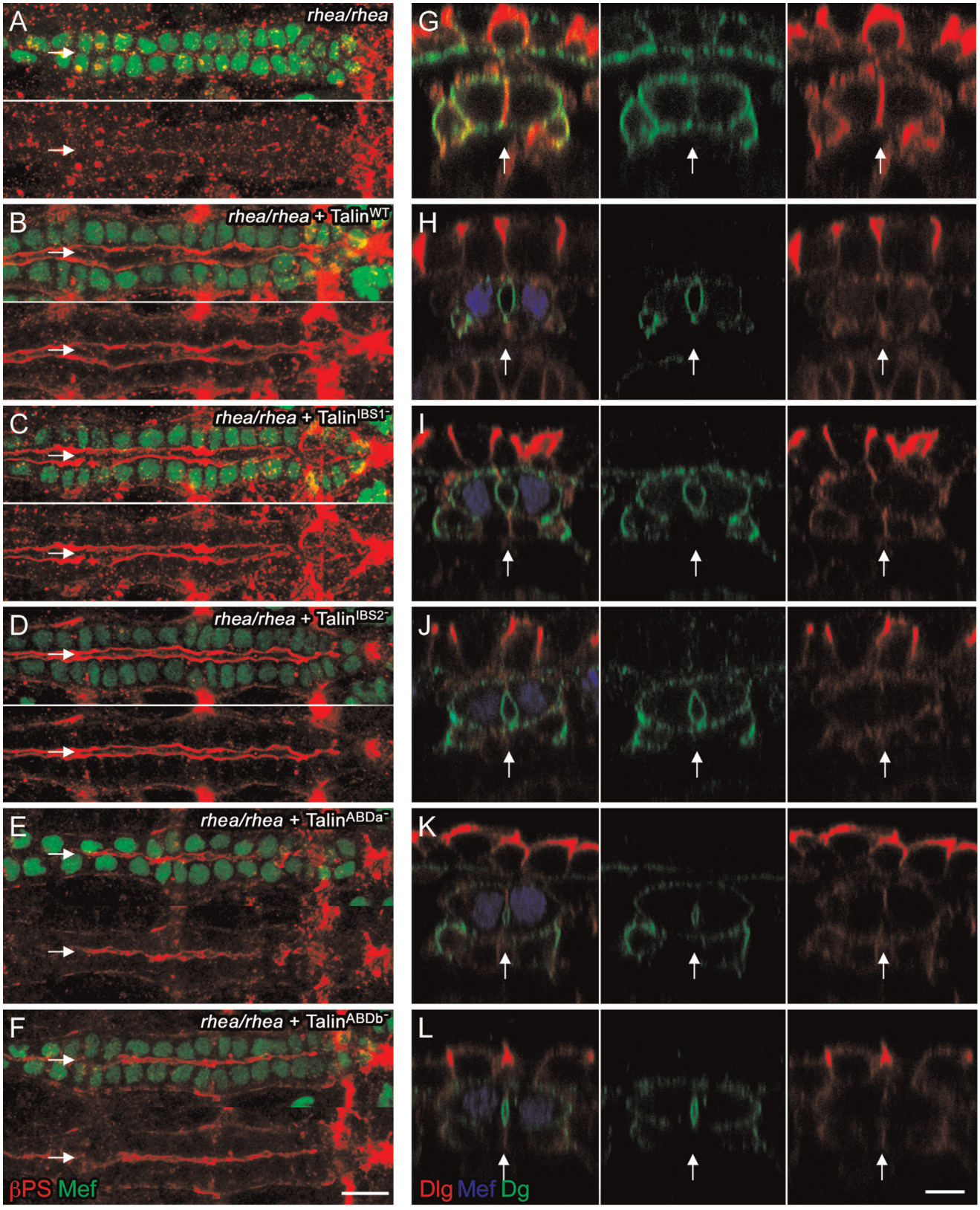


Fig. 6. Rescue of lumen formation using integrin or actin binding deficient Talin. CB position and βPS1 localization in stage 17 zygotic *rhea* null embryos ubiquitously expressing no rescue construct (A), wildtype Talin (B), Talin with disrupted integrin binding through IBS1 (C) or IBS2 (D), or Talin with abrogated actin binding (E and F). In contrast to the tight juxtaposition of the contralateral CB in zygotic *rhea* null embryos (arrow, A), embryos rescued with Talin^{WT}, Talin^{IBS1-}, or Talin^{IBS2-} maintained an open space between the CB rows and continuous distribution of βPS1 along the CB apical surface (arrow, B). *rhea* embryos expressing Talin^{ABDa-} or Talin^{ABDb-} were able to stabilize βPS1 along the apical surface, but had a reduced space between CB rows (arrow, E and F). All βPS label was imaged using identical settings. Transverse images of the heart are immunolabelled with Dg (green) to outline the cardioblasts and luminal surface and Dlg (red) to identify midline junctional domains between opposing cardioblasts (G–L). While *rhea* homozygotes display only a Dlg-rich junctional domain between opposing cardioblasts (arrow, G), mutants rescued with Talin^{WT}, Talin^{IBS1-}, or Talin^{IBS2-} have an open Dg-lined lumen and junctional regions restricted to small dorsal and ventral contact sites (above vertical arrows in H–J). Compared to the wildtype rescue, *rhea* mutants expressing Talin^{ABDa-} or Talin^{ABDb-} exhibit a small luminal domain and enlarged dorsal and ventral junctional regions (K and L). Calibration: 10 μm in A–F, 5 μm in G–L.

Table 1
Luminal width of the heart proper in Talin rescues

Genotype	*rhea*^{79}/+	*rhea*^{79}	*rhea*^{79}+WT	*rhea*^{79}+IBS1^{-}	*rhea*^{79}+IBS2^{-}	*rhea*^{79}+ABDa^{-}	*rhea*^{79}+ABDb^{-}
Lumen (μm)	***3.47***	1.85	***3.40***	***3.11***	***3.23***	2.08	2.32
Standard error	±0.21	±0.16	±0.22	±0.19	±0.22	±0.19	±0.21
# of embryos	24	23	26	23	20	27	21

*Lumen width was measured as the distance between contralateral Tinman-positive cardioblast nuclei. Measurements were taken at the middle of each full segment within the heart proper and averaged for each embryo. Scores which are significantly greater than that for homozygous *rhea* mutants are italicized and bolded (Mann–Whitney *U* test, $p < 0.0001$).

localization of βPS1 integrin, Talin^{ABDa-} and Talin^{ABDb-} were only able to partially rescue the lumen phenotype (Fig. 6K and L). In contrast to *rhea* mutants (Fig. 6G), rescued embryos were able to establish a Dg-rich midline luminal domain (green above arrow in Fig. 6K and L). However, compared to *rhea* mutants rescued with a wildtype Talin transgene (Fig. 6H), the luminal space was markedly reduced and the dorsal and ventral midline junctional domains were expanded (Fig. 6K and L, Table 1). Elevated levels of Talin^{ABDa-} or Talin^{ABDb-} expression did not alter the degree of rescue, suggesting this was not a threshold effect (Fig. 2H and I in Supplementary material). Taken together, these data suggest that although direct Talin–actin linkage is not required to position the luminal domain, it is essential for the CB cytoskeletal dynamics required to generate the crescent-shaped morphology and to develop an open lumen.

Discussion

The experiments reported here establish an essential function for the integrin adapter Talin in the assembly of the *Drosophila* embryonic heart. During the CB migratory phase preceding tubulogenesis, Talin localizes along the CB apical surface, immediately ventral to the leading edge which extends towards the dorsal midline. As this Talin rich domain persists throughout embryonic heart assembly, eventually surrounding the lumen of the open cardiac tube, we term this surface the pre-luminal domain. Talin is essential for the dynamic cell morphology and the leading edge features that characterise collective cardial cell migration. Furthermore, following migration, Talin is required to enclose a continuous lumen between the bilateral CB rows.

Talin instructs cardioblast polarity to orient lumen formation

Analysis of late stage hearts in *rhea* zygotic mutants reveals that Talin is essential to correctly orient the CB polarity such that a continuous lumen is enclosed along the midline. In wildtype, many membrane receptors including Robo, Dg, Unc5, and Syndecan accumulate along the luminal domain (Medioni et al., 2008; Albrecht et al., 2011; Knox et al., 2011). E-cadherin, Dlg, and other cell–cell adhesion factors are restricted to cell contact points immediately dorsal and ventral to the lumen and to the lateral cell domains between ipsilateral CBs (Santiago-Martinez et al., 2008; Medioni et al., 2008). As evidenced by Robo and Dg immunolabeling experiments, the midline luminal domain is absent or, at best, is discontinuous along the midline in *rhea* mutant embryos. However, the Robo and Dg enriched luminal domains are not completely absent in null *rhea* homozygotes, but are found ectopically along lateral membranes between ipsilateral CBs. Robo's ligand, Slit, is also detected within these ectopic lumina. Similar ipsilateral Slit and Robo accumulations were observed in embryos mutant for the integrin subunit genes *scab* (αPS3) or *mys* (βPS1; Vanderploeg et al., 2012). Thus, the expanded Dlg-rich adhesive contact observed in *rhea* null embryonic hearts is consistent with a model in which integrins and Talin instruct the localization of Slit and Robo. These cues are essential to orient the lumen and to restrict the adhesive regions. In the absence of Talin, other components of the luminal structure, including Dg and the Slit–Robo complex, can self-assemble and create non-adherent luminal domains. However, proper midline positioning of the lumen requires Talin function.

Multiple Talin domains are required for polarization and lumen formation

Using an array of Talin transgenes previously shown to modify integrin adhesion strength and actin recruitment (Ellis et al., 2011; Franco-Cea et al., 2010), we assessed and compared the importance of these Talin-dependent processes. Binding of Talin's IBS1 to a membrane proximal NPxY motif on the β-integrin tail induces conformational changes within the integrin dimer, activating it and increasing the affinity for ECM ligands (Tadokoro et al., 2003; Wegener et al., 2007). Integrin activation is likely required prior to Talin IBS2 binding (Rodius et al., 2008), an interaction which promotes a strong and stable integrin–cytoplasmic adhesome linkage (Ellis et al., 2011). Our data indicates that either of Talin's two integrin binding sites are sufficient to promote CB morphogenesis and heart tube assembly. The ability of the heart to form in the presence of only IBS1 or IBS2 suggests that strong, long-lasting integrin-mediated adhesions are unnecessary. This idea is reinforced by the late accumulation of CAP (data not shown), a protein recruited to more mature muscle adhesions (Bharadwaj et al., 2013). It is likely that transient adhesions are sufficient for lumenogenesis. It remains possible that an essential role for either IBS1 or IBS2 is masked by the perdurant maternal Talin in zygotic mutants. However, the functional redundancy of these domains is consistent with *in vitro* and *in vivo* studies suggesting that a subset of Talin functions can be fulfilled by either IBS domain (Calderwood et al., 2002; Wegener et al., 2007; Tremuth et al., 2004; Tanentzapf and Brown 2006; Ellis et al., 2011; Klapholz et al., 2015).

Talin links integrins to the actin cytoskeleton both directly through an actin binding domain, or indirectly through recruitment of actin regulators such as Vinculin. Bond force studies of the C-terminal ABD suggest that although the ABD-actin linkage is direct, it is a weak bond which likely relies on additional direct or indirect Talin–actin linkages to form a strong and stable connection (Jiang et al., 2003). Supporting this, Talin^{ABD} is essential for morphogenetic processes which rely on transient and dynamic integrin–actin linkages, but it is at least partially dispensable for longer-lasting adhesions which are likely stabilized by indirect Talin–actin interactions through Vinculin (Franco-Cea et al., 2010; Klapholz et al., 2015). Our studies demonstrate that *Drosophila* heart development is sensitive to disruptions in Talin's C-terminal ABD, which implicates cytoskeletal reorganization as a key process downstream of integrins during tubulogenesis. Supporting this, expression of constitutively active Diaphanous or dDAAM, formin proteins which promote actin polymerization, induced ectopic lumina similar to those we have characterized in *rhea* mutants (Vogler et al., 2014). These data are consistent with Talin promoting CB morphogenesis and lumen

formation through direct, but dynamic actin linkages and suggest that formins may act downstream of Talin in apicalizing lumen formation.

An emerging hierarchy in lumen formation

To date, most studies on the *Drosophila* embryonic heart have focused on cell surface factors including receptors and their respective ligands; few studies have moved into the cell to establish the downstream signaling pathways involved. Insights into *in vitro* models suggest that polarity pathways and vesicle trafficking will be informative areas of study. For example, in the MDCK cyst model, the small GTPases Rab8a and Rab11a coordinate with the exocyst complex to deliver luminal factors to the pre-luminal initiation site (Bryant et al., 2010). It remains to be determined whether similar exocytosis or secretion mechanisms are required for *Drosophila* heart lumen initiation or expansion. Furthermore, although it is unclear which classical apical polarity proteins are conserved in the *Drosophila* heart, epithelial and endothelial models suggest that the Cdc42–Par6–aPKC complex is a conserved master regulator of tube formation in both vertebrates and flies (e.g. Bryant and Mostov, 2008; Sacharidou et al., 2010; Pirraglia et al., 2010; Qi et al., 2011; Jones and Metzstein, 2011). Indeed, *Drosophila* heart tubulogenesis fails in embryos with heart specific inhibition of Cdc42 and expression of activated Cdc42 results in lateral lumina reminiscent of those we characterized in *rhea* homozygotes (Vogler et al., 2014; Swope et al., 2014). We envision a mechanism of heart tubulogenesis in which Talin provides instructive cues to the vesicle trafficking and polarity networks that target luminal factors and inhibit the assembly of cell–cell adhesion structures within the pre-luminal domain.

Acknowledgments

We thank the following investigators for providing *Drosophila* strains: B. McCabe (dMEF-Gal4), T. Millard (UAS–moesin–mcherry), R. Schulz (TUP-GFP), and G. Tanentzapf (Talin rescue strains, $rhea^{2}$, $rhea^{79}$, mys^{XG43}, and yw; hsFLP; Dr/TM3, Sb). All other stocks were provided by the Bloomington Stock Center, the Vienna *Drosophila* RNAi Centre, and the *Drosophila* Genetic Resource Centre (Kyoto). Polyclonal antibodies were generously supplied by M. Beckerle (Pinch), A. Kolodkin (CAP), and Z. Lai (Zfh1). The pGEX-5X-1-Dg-cyto plasmid was kindly provided by W. Deng. Research was funded by OGS, NSERC CGS-D (JV), and NSERC (466512012).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.04.024.

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